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Reducing Salt from Autolysate of Fermented Mung Bean (*Phaseolus radiatus* l.) using Diafiltration-Nanofiltration (DF-NF) Mode for Quality Improvement of Savory Flavor Product

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Abstract

Diafiltration-Nanofiltration (DF-NF) mode of fermented mung beans (Phaseolus radiatus L.) autolysate by Rhizopus sp.-PL19 and Aspergillus sp.-K3 to recover L-glutamic acid as savory fraction with salt concentration relating with organoleptic aspect has been done. DF-NF mode was performed by adding pure water to autolysate feed in 0/3500, 700/3500, 1400/3500, 2100/3500, and 2800/3500 mL/mL as Number of diavolume (Nd) of 0, 0.2, 0.4, 0.6, and 0.8 at DF flow rate of 35.66 and 35.5 mL/minute at pump motor frequency of 20 Hz (~ 7.5 L/minute), room temperature, and pressure of 20 bar for 155.3 and 197.6 minutes, respectively. The result indicated that DF-NF mode on autolysate-Rhizopus sp.-PL19 and autolysate-Aspergillus sp.-K3 was able to reduce salt at Nd of 0.2, namely 20 and 4.76 %, respectively. High Nd gave high salt reduction in retentate. At the optimal Nd (0.2) was resulted compositions of retentate-Rhizopus sp.-PL19 and retentate-Aspergillus sp.-K3 with concentrations of salt of 0.106 and 0.53 %, N-amino of 7 and 7 mg/mL, and L-glutamic acid as savory fraction of 0.5063 and 0.8437 % (total protein), respectively, while at permeate gave flux value 70 and 51.23 L/m2.hour, and concentrations of salt 0 and 0.1325 %, N-amino 3.5 and 1.4 mg/mL, and L-glutamic acid 0.6375 and 0.3188 % (total protein), respectively.

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Keywords: Diafiltration-Nanofiltration (DF-NF); Number of Diavolume (Nd); L-glutamic acid; retentate (concentrate); permeate; salt

1. Introduction

Mung beans (Phaseolus radiatus L.), which have been a staple part of diets for thousands of year are widely consumed in East Asia countries (China, Korea, Japan and Taiwan), and South-East Asia countries (Vietnam, Thailand, Malaysia, Singapore, and Indonesia). Mung beans have been the potential sources as functional and valuable ingredient in prepared food systems. To reduce the consumption of animal protein products for health and

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economic reasons, there is a possibility that vegetable protein will constitute and contribute a much higher proportion of the human diet in the future[1]. One of the alternatives to manufacture prepared mung bean is an application of brine fermentation on mung bean as solid substrates using inoculum of Rhizopus sp.-PL19 and Aspergillus sp.-K3 as savory (umami) ingredient and seasoning agents. The transferring and transformation of salt into solid substrate media is mainly gradual, in which its rate depends on many factors, such as the variety of mung beans used, mung beans-salt-inoculum proportion, fermentation condition (temperature and time), kind and concentration of inoculum, etc[2]. The product generated through brine fermentation process was is still crude fermented mung bean containing rich amino acids with the salt concentration requiring further process steps, such as extraction, homogenization, first and second filtrations, membranes of microfiltration (MF) and nanofiltration (NF). Amino acids have been well-known as precursor of savory flavor contained in fermentation products, such as ketchup, tauco (Indonesia), miso and katsuobushi (Japan), bagoong and tao-si (Philiphine), meju (Korea), and prahoc (Cambodia). These amino acids were generally produced by using inoculums of Aspergillus sp. and Rhizopus sp. as microorganism in resulting enzyme which will convert and degrade components in mung beans to volatile and non-volatile compounds in forming savory (umami) taste. The high salt concentration of crude fermented mung bean product will limit and effect its use in prepared food products[3].

Nanofiltration (NF) is a relatively new class of membrane process which is considered to be bridge between Ultrafiltration (UF) and Reverse Osmosis (RO) processes. The term NF is derived from the fact that these molecular weight cut-off (MWCO) values correspond to hypothetical pores of ~ 1 nm. This new class of membranes has a very low pore size of 1 – 3 nm or approximately ~ 50 Da. and needs a moderately driven pressure ranging from 5 to 50 bars[4,5]. The NF generates a retentate and permeate. The NF retentate has a higher concentration of some salts and organic compound MWCO in the 200 – 500 Dalton (Da.) range or the pores of the membrane than the feed. The NF permeate will comprise micro solutes and organic compound that have passed freely from the feed through the NF membrane. Hence, membrane selectivity is not based only on the sieve effect and molecular size, but also on the charge effect[6]. Diafiltration-Nanofiltration (DF-NF) mode is a technique that uses NF membrane to partially or completely remove, eliminate or replace the concentration of salts from fluids containing proteins, peptides, nucleic acids, and other biomolecules by adding fresh water (dialysate) at the approximated same rate as permeate withdrawn, continuing NF operation, keeping constant feed or retentate (concentrate) volume, increasing the macrosolutes concentration and decreasing and/or reducing micro solute concentration to overcome low permeate fluxes at high concentration or to get better permeable species with the goal of washing out. The process selectively uses permeable (porous) membrane to separate the components of fluids based on their molecular size. An NF membrane retains microsolutes with a molecular weight (MW) in the range 200 – 500 Da. that are larger than the pores of the membrane while smaller molecules (salts, solvents and water), and which are 100 % permeable, freely pass through the membrane. DF-NF mode has been widely applied in recovering, separating and concentrating valuable compounds in the agro-food, dairy, pharmaceutical, biotechnology, and biochemistry industries due to low energy consumption, no phase change, and its operation at normal temperatures[7].

The goal of this experiment was to decrease salt content in retentate (concentrate) as a result of DF-NF mode on autolysates of fermented mung bean by Rhizopus sp.-PL19 (Suspension A) and Aspergillus sp.-K3 (Suspension B) at flow rate ~ 7.5 L/minute, room temperature (~ 23 – 25 oC) and pressure 20 bar with various fresh water to feed or retentate ratio (Nd).

2. Materials and Methods

2.1. Material and Equipment

Raw materials used in this work were concentrate-Rhizopus sp.-PL19 (Suspension A) and concentrate-Aspergillus sp.-K3 (Suspension B) purified by means of MF membrane of 0.2 μm and concentrated through NF membrane, respectively, 2 (two) sheets of MF membrane of 0.2 μm, 2 (two) sheets of NF thin film composite membrane (NF-45-PE, diameter of membrane 20 cm and effective area of 0.036 m^2), fresh water (RO water) and chemicals. All chemicals were reagent grade obtained commercially.

Equipments utilized in this work were fermentation system in laboratory scale (local, sieve (Retsch, Germany), autoclave (Cheng Yi, LS-50L, China), shaker (Memmert, Germany), homogenizer (Ultra-Turrax, Ika Labortechnik,

T50, Jane & Kunkel, Germany), MF/UF/NF/HP membrane module (LabUnit M20, Danish Separation Systems AS, Nakskov, Denmark) equipped by Positive Displacement Pump Rannie 25.38 (tangential flow rate of 3.5 – 15 L/minute)[5], Salinity Meter (ATAGO, Japan), HPLC, and spectrophotometer UV-Vis.

2.2. Experimental Design

This experiment was performed using various fresh water to retentate (concentrate) ratio as Diavolume Number (Nd) of 0.2, 0.4, 0.6, 0.8 and 1 at fixed condition, such as flow rate 7.5 L/minute, room temperature (~ 23 - 25 oC) and pressure 20 bar through NF membrane module. The samples were analyzed in terms of N-amino (Cu method)[8], salt (Salinometer), and L-Glutamic Acid (Boehringer method)[9]. Investigation on performance of NF membrane was permeate flux value.

2.3. Process Step

The sample tank of the membrane module was filled with the suspension A to about one-third capacity (3,500 mL). The suspension A in feed tank is subsequent pumped tangentially by means of a fixed pump motor frequency (20 Hz) through a pre-filtered in 200 μm filter, heat exchanger system, and into a membrane module (plate & frame) mounted vertically for 5 minutes at room temperature, tangential flow rate of 7.5 L/minute and pressure of 20 bar until the permeate contained colorless liquid. Retentate (concentrate) or feed is recirculated to the feed tank to keep an effectively constant concentration. Diafiltration-Nanofiltration (DF-NF) technique was performed in the continuous mode, in which the permeate flux is compensated by an equal input of RO water (32 mL/minute). During DF-NF, pure water (RO water) is introduced into the suspension feed tank while permeate is removed and eliminated from the suspension feed tank through the same membrane surface. In this case, microsolutes in the feed or retentate (concentrate) are progressively decreased and removed as permeate flux. In this experiment, it was conducted a variety of RO water to feed or retentate (concentrate) volumes ratio 0/3500, 700/3,500, 1,400/3,500, 2,100/3,500 and 2,800/3,500 mL/mL (Nd 0, 0.2, 0.4, 0.6 and 0.8), respectively. Retentate (concentrate) and permeate were regularly sampled and recorded for analysis of their salt, N-amino and L-Glutamic Acid concentrations, respectively. The same procedure was carried out for Suspension B. At the end of each work, the membranes were thoroughly flushed with RO water. The membranes were then cleaned in place using 4 % NaOH solution before storing in 1 % Sodium Azide till the subsequent run. Flow diagram of DF-NF mode on autolysates of mung bean fermented by Rhizopus sp.-PL19 (Suspension A) and Aspergillus sp.-K3 (Suspension B) is represented in Figure 1.

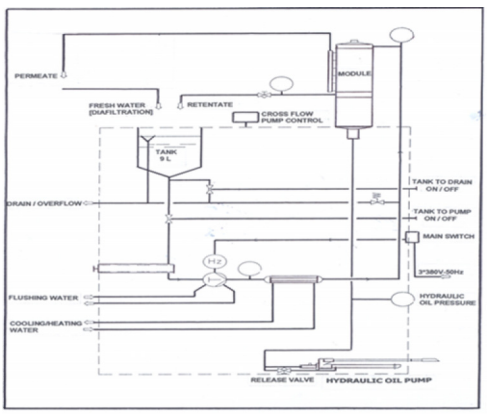
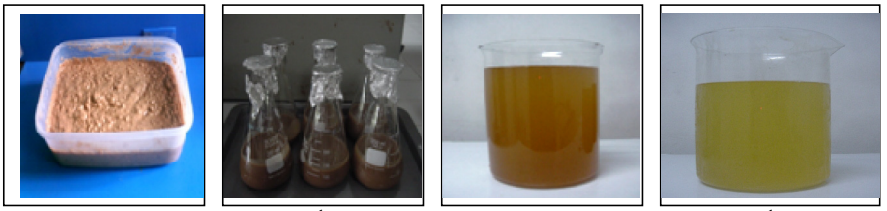


Figure 1. Flow diagram of DF-NF mode on autolysates of mung bean fermented by Rhizopus sp.-PL19 (Suspension A) and Aspergillus sp.-K3 (Suspension B)[5]

3. Results and Discussion

3.1. Characteristic of autolysates of mung bean fermented by Rhizopus sp.-PL19 (Suspension A) and Aspergillus sp.-K3 (Suspension B)

Autolysate of fermented mung bean is a result of autolysis process on mung bean through brine fermentation by inoculum of Rhizopus sp.-PL19 and Aspergillus sp.-K3 at 50 °C and pH 5.5 for 16 hours. Autolysis is conducted to increase savory fraction content, especially L-Glutamic acid as savory flavor fraction through activity of protease enzyme. To separate savory fraction in autolysate is performed by using a MF membrane of 0.2 µm so it produces retentate (concentrate) and permeate. The permeate is then concentrated through NF membrane to generate retentate (concentrate) as feed in further salt DF mode. Concentrate-Aspergillus sp.-K3 (Suspension B) is a brownish yellow clear liquid, umami and salty tastes. Concentrate-Rhizopus sp.-PL19 (Suspension A) has a darker liquid than concentrate-Aspergillus sp.-K3 (Suspension B). This liquid color relates with kind of inoculum, fermentation time, and process condition of autolysis, in which the presence of heat formed brown pigment as a Maillard reaction between monosaccharide and amino acids[10]. Fig. 2 showed fermented mung bean (a), autolysate of fermented mung bean (b), concentrate-Rhizopus sp.-PL19 (Suspension A) (c) and concentrate-Aspergillus sp.-K3 (Suspension B) (d) as a result of NF as feed in DF-NF mode.



a b c d

Figure 2. Fermented mung bean for 18 weeks (a), autolysat (b), concentrate-*Rhizopus* sp.-PL 19 (c) and concentrate-*Aspergillus* sp.-K3 as feed in DF-NF mode (d)

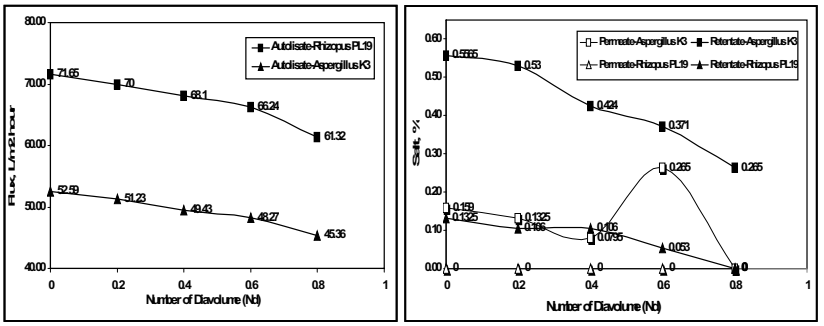
Concentrate composition as feed in salt DF-NF mode demonstrates that total solid concentration (0.7937 %) of Suspension A was lower than that in Suspension B (2.8531 %). Total solid is an accumulation of all components in Suspension both soluble and non-soluble solids. This concentrate is a result of autolysate concentrating through multi filtration (MF and NF) because soluble components have particle size than 1 - 10 nm[11]. The important components in suspension as savory fraction are amino acids as N-Amino and dissolved protein. N-Amino concentration in Suspension A (0.7 mg/mL) which is higher than that in Suspension B (0.56 mg/mL) display its presence of relationship between initial suspension (crude fermented mung bean), kind and inoculum proteolytic activity, multi filtration process, and property and characteristic of amino acids in suspension.

Non-essential amino acids as N-amino in concentrate using both same kinds of fungus and passing as permeate in MF membrane of 0.2 µm is dominated by L-Glutamic acid having molecular weight of 147 with particle size of 0.01 – 0.1 µm[12], besides aspartic acid and proline. Whereas, dominant essential amino acids are lysine, leucine, arginine, isoleucine and phenilalanine. Other amino acids is produced in concentration of 0.164 – 0.289 %, such as serine, glicine, histidine, alanine, tyrosine, valine, methionine, cystine and phenilalanine[11]. Based on salt content, salt in Suspension A (0.1325 %) are lower than that in Suspension B (0.5565 %). This difference are not only caused by its passing grade in before process (multi filtration, MF and NF), but also by difference in initial Suspension (crude fermented mung bean) concentration relating to brine fermentation. This higher salt concentration is a reason why DF-NF mode is operated.

3.2. Effect of DF-NF mode condition on flux and compositions

Permeate flux value and salt

Relationship between Number of diavolume (N_d) and permeate flux value on both kinds of autolysate suspensions are demonstrated in Fig. 3a. Increase of N_d will decrease permeate flux at both autolysates. Permeate flux of Suspension A at N_d 0, 0.2, 0.4, 0.6, and 0.8 are 71.65, 70, 68.1, 66.24 and 61.32 L/m^2.hour, with cumulative time are 0, 14.4, 43.3, 87.8 and 155.3 minutes, respectively, whereas permeate flux of Suspension B at the same condition are 52.59, 51.23, 49.43, 48.27, and 45.36 L/m^2.hour with cumulative time are 0, 19.8, 59.9, 120.1, and 197.6 minutes, respectively. Decreasing in permeate flux value is caused by feed flow rate which flows tangentially on membrane surface in which this tangential flow sweeps solute particles to bulk solution (feed/retentate). Besides, same flow rates between fresh water to feed tank and pure solvent passing via NF membrane (permeate) will cause constant feed/retentate volume. This condition causes permeate flux to be constant. Permeate flux value of Suspension A is higher than that of Suspension B. This case is possibility caused by lower concentration of total solid in Suspension A (0.7937 %) than that in Suspension B (2.8531 %).



(a) (b)

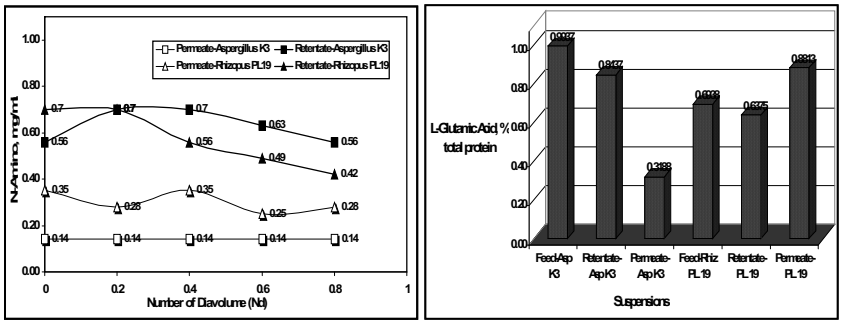
Figure 3. Effect of N_d on permeate flux (a) and salt content (b) in DF-NF mode of concentrate-*Rhizopus* sp.-PL19 and concentrate-*Aspergillus* sp.-K3 at flow rate 7.5 L/minute, room temperature and pressure of 20 bar.

The overall processes, more and more high N_d at both kinds of Suspensions drop salt concentration both in retentate and permeate, except at N_d 0,6 will increase salt concentration in permeate A (0.265 %). At N_d of 0, 0.2, 0.4, 0.6 and 0.8 for both retentates of suspension A and B are 0.1325, 0.106, 0.106, 0.053 and 0 %, and 0.5565, 0.53, 0.424, 0.371 and 0.265 %, respectively, while salt concentration in permeates of suspension A and B are 0 %, and 0.159, 0.1325, 0.0795, 0.0265 and 0 %. In this N_d 0,2, salt concentration in retentates are relating to organoleptic aspect, namely 0.106 % (suspension A) and 0,53 % (suspension B). Salt reduction in retentate with N_d 0,2 for Suspensions A and B compared with feed are 20 and 4,76 %, respectively. This condition is caused not only by difference in initial feed concentration but also by interaction of process condition and interaction among components in suspension.

Decreasing salt content in retentate occurs due to DF-NF system, in which fresh water added to feed tank with constant volume (3,500 mL) become more and more large so salt solubility in Suspension will become more and more high, although salt particle size is much larger than pore size of NF membrane (± 0.001 μm)[12]. Although, it occurs a salt reduction, but particles salt retained in retentate are much more than that passed as permeate for all N_d treatment, indicated in Figure 3b. Salt is a strong electrolite dissociating almost perfect to form charge particles (ion), but this electrostatic bond can be separated by pure water as the best solvent, in which attractive force between water pole and Na^+ and Cl^- overcomes attractive force both ions so salt is able to solve with high stability[13].

N-amino and L-Glutamic Acid

Increasing in N_d produces diafiltrate with higher N-amino and dissolved protein in retentate than in permeate, as displayed in Fig. 4a and 4b. DF-NF mode at N_d 0, 0.2, 0.4, 0.6 and 0.8 on Suspension A results N-Amino in retentate are 0.7, 0.7, 0.56, 0.49 and 0.42 mg/mL, whereas N-Amino in permeate are 0.35, 0.28, 0.35, 0.25 and 0.28 mg/mL, respectively. DF-NF mode at N_d 0, 0.2, 0.4, 0.6 and 0.8 on Suspension B results N-Amino in concentrate are 0.56, 0.7, 0.7, 0.63 and 0.56 mg/mL, while N-Amino in permeate are 0.14, 0.14, 0.14, 0.14 and 0.14 mg/mL, respectively. DF-NF mode yields higher concentration of N-Amino in retentate B than retentate A for all N_d treatments. More and more high N_d decay N-Amino in retentate of suspension A at N_d 0.4, 0.6 and 0.8, but for retentate B, dropping N-Amino is started at N_d 0.2. Both these kinds of Suspensions generate higher concentration of N-Amino in retentate than in permeate for all N_d treatments. Decreasing N-Amino is relating with an increase of N_d. This case is probability caused by more and more much Amino Acids particle passing in permeate, although the overall amino acids concentration in retentate is higher than in permeate for each N_d. Larger size of Amino Acids particle (0.008 – 0.1 μm) than pore size of NF membrane (~ 0.001 μm)[12] causes much more Amino Acids retained on membrane surface so Amino Acids concentration in retentate are higher than in permeate for both kinds of Suspensions. Amino Acids have high solubility in water[13].



(a) (b)

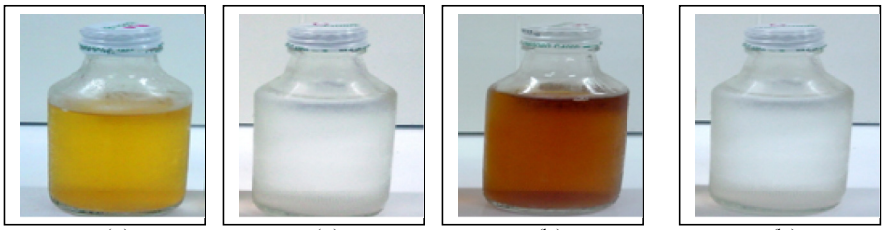
Figure 4. Effect of N_d on N-Amino (a) and L-Glutamic Acid contents (b) in feed, retentate and permeate via DF-NF mode of concentrate-*Rhizopus* sp.-PL19 and concentrate-*Aspergillus* sp.-K3 at flow rate 7.5 L/minute, room temperature and pressure of 20 bar

This contributes more much mount of Amino Acids to pass through membrane as permeate relating with more and more high flow rate of fresh water to constant feed volume (3,500 mL). Other possibility showed that Amino Acids particles are accumulated at the membrane surface till the formed cake layer at the membrane surface (fouling)[14]. For suspension A, this is initiated at N_d 0.4, but for suspension B is started at N_d 0.2. Difference in total solids concentration for both suspensions cause a different fouling intensity. DF-NF system reduce not only salt, but also has an important role in separation of N-Amino, in which suspension B indicates more effective separation than suspension A because membrane has better performance marked by profile of N-Amino concentration in retentate and permeate.

L-Glutamic Acid is one of the non-essential Amino Acids from all Amino Acids (essential and non-essential) generated naturally through brine fermentation on mung bean by *Rhizopus* sp. PL19 and *Aspergillus* sp.K3 and is a precursor of non-volatile as source of savory (umami) taste**.** Based on investigation of organoleptic aspect on salt content in concentrate, N_d 0.2 is accepted limitation of N_d in which salt reduction in suspension A and B are 20 and 4.76 %, respectively. The effect of salt reduction in diafiltrate on L-Glutamic Acid concentration will influence on all savory taste intensities. Difference in L-Glutamic Acid concentrations before DF (N_d 0), at the best N_d (0.2) and feed. It occurs a difference in L-Glutamic Acid concentration for both suspensions. At diafiltrate A, L-Glutamic Acid pass more much in permeate (0.8813 % total protein) than that retained at retentate (0.6375 % total protein), whereas at diafiltrate B in DF-NF system is able to retain L-Glutamic Acid in retentate (0.8437 % total protein)

when compared to pass in permeate (0.3188 % total protein). Thus, it had been occured a decline of L-Glutamic Acid in concentrate in suspension A (15 %). It another case, at suspension B, it had been occured an increase of L-Glutamic Acid in concentrate (0,5 %) when compared to suspension feeds of A and B, namely 0.9937 % (total protein) and 0.6938 % (total protein), respectively. These conditions are possibility not only caused by particle size of L-Glutamic Acid ranging 0.01 – 0.1 μm with MW 147 which is larger than pore size of membrane 40 – 2000 nm or range of 0.001 – 0.01 μm, but also by membrane performance of NF, such as interaction between flow rate 7.5 L/minute and pressure of 20 bar, and fresh water to suspension feed ratio (N_d 0.2). Another possibility is its presence of difference on initial suspension (feed) in which initial concentration of L-Glutamic Acid (0.6938 % total suspension) in suspension A is lower than L-Glutamic Acid (0.9937 % total suspension) in suspension B affecting on recovery of L-Glutamic Acid in diafiltrate.

Figures $5a_1$, $5a_2$, $5b_1$, and $5b_2$ indicate retentate and permeate products of suspensions of A (*Rhizopus* sp.-PL19) and B (*Aspergillus* sp.-K3) under the best N_d (0.2). From all runs, DF-NF mode in suspensions A and B under the best conditions relating with organoleptic aspect are at N_d 0.2 and 0.2, respectively.



(a_1) (a_2) (b_1) (b_2)

Figure 5. Retentate (a_1) and permeate (a_2) as low salt savory fraction from *Rhizopus* sp.-PL19, and retentate (b_1) and permeate (b_2) as low salt savory fraction from *Aspergillus* sp-K3.

4. Conclusions

The result of experiment showed that DF-NF mode of concentrate-Rhizopus sp.-PL19 (Suspension A) and concentrate-Aspergillus sp.-K3 (Suspension B) was able to reduce the optimal salt content at Nd 0.2, namely 20 % and 4.76 %, respectively. High Nd gave high salt reduction in retentate (concentrate). At the best Nd (0.2) gave composition of concentrate-Rhizopus sp.-PL19 and concentrate-Aspergillus sp.-K3, such as salt of 0.106 and 0.53 %, N-Amino 7 and 7 mg/mL, and L-Glutamic Acid as savory fraction of 0.5063 and 0.8437 % (total protein), respectively, while permeate as side product gave permeate flux values, 70 and 51.23 L/m2.hour, and concentrations of salt of 0 and 0.1325 %, N-Amino of 3.5 and 1.4 mg/mL, and L-Glutamic acid of 0.6375 and 0.3188 % (total protein), respectively. At the best Nd (0.2) occurs a decrease of L-Glutamic Acid in retentate (concentrate) of Suspension A (15 %), while it occurs an increase of L-Glutamic Acid in retentate (concentrate) (0.5 %) when compared to feed.

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